

<https://helda.helsinki.fi>

Fungal feruloyl esterases: Functional validation of genome mining based enzyme discovery including uncharacterized subfamilies

Dilokpimol, Adiphol

2018-03-25

Dilokpimol , A , Mäkelä , M R , Varriale , S , Zhou , M , Cerullo , G , Gidijala , L , Hinkka , H T , Brás , J L A , Jütten , P , Piechot , A , Verhaert , R , Hilden , S K , Faraco , V & de Vries , R 2018 , ' Fungal feruloyl esterases: Functional validation of genome mining based enzyme discovery including uncharacterized subfamilies ' , New Biotechnology , vol. 41 , pp. 9-14 . <https://doi.org/10.1016/j.nbt.2017.11.004>

<http://hdl.handle.net/10138/307191>

<https://doi.org/10.1016/j.nbt.2017.11.004>

cc_by_nc_nd

acceptedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

**Fungal feruloyl esterases: functional validation of genome mining based enzyme discovery
including uncharacterized subfamilies**

**Adiphol Dilokpimol^{a,1}, Miia R. Mäkelä^{b,1}, Simona Varriale^{c,1}, Miaomiao Zhou^{a,g}, Gabriella
Cerullo^c, Loknath Gidijala^d, Harri Hinkka^b, Joana L.A. Brás^e, Peter Jütten^f, Alexander
Piechot^f, Raymond Verhaert^d, Kristiina S. Hilden^{b,*}, Vincenza Faraco^{c,*}, Ronald P. de
Vries^{a,*}**

^aFungal Physiology, Westerdijk Fungal Biodiversity Institute & Fungal Molecular Physiology,
Utrecht University, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

^bDivision of Microbiology and Biotechnology, Department of Food and Environmental Sciences,
University of Helsinki, Helsinki, Finland

^cDepartment of Chemical Sciences, University of Naples “Federico II”, Complesso Universitario
Monte S. Angelo Via Cintia, 4 IT-80126, Naples, Italy

^dProteoNic B.V., J.H. Oortweg 19-21, 2333 CH Leiden, The Netherlands

^eNZYTech B.V., Estrada do Paço do Lumiar, Campus do Lumiar, Edifício E – 1º andar, 1649-
038 Lisboa, Portugal

^fTaros Chemicals GmbH & Co. KG, Emil-Figge-Str. 76a, 44227 Dortmund, Germany

^gCurrent address: Academie voor de Technologie van Gezondheid en Milieu, Avans Hogeschool,
Lovensdijkstraat 61, 4818 AJ Breda, The Netherlands

*Corresponding authors.

E-mail addresses: r.devries@westerdijkinstituut.nl (R.P. de Vries), kristiina.s.hilden@helsinki.fi (K.S. Hilden); vincenza.faraco@unina.it (V. Faraco).

¹Equal contribution.

Abstract

Feruloyl esterases (FAEs) are a diverse group of enzymes that specifically catalyze the hydrolysis of ester bonds between a hydroxycinnamic (e.g. ferulic) acid and plant poly- or oligosaccharides. FAEs as auxiliary enzymes significantly assist xylanolytic and pectinolytic enzymes in gaining access to their site of action during biomass saccharification for biofuel and biochemical production. A limited number of FAEs have been functionally characterized compared to over 1,000 putative fungal FAEs that were recently predicted by similarity-based genome mining, which divided phylogenetically into different subfamilies (SFs). In this study, 27 putative and six characterized FAEs from both ascomycete and basidiomycete fungi were selected and heterologously expressed in *Pichia pastoris* and the recombinant proteins biochemically characterized to validate the previous genome mining and phylogenetical grouping and to expand the information on activity of fungal FAEs. As a result, 20 enzymes were shown to possess FAE activity, being active towards *p*NP-ferulate and/or methyl hydroxycinnamate substrates, and covering 11 subfamilies. Most of the new FAEs showed activities comparable to those of previously characterized fungal FAEs.

Keywords

Feruloyl esterase, ferulic acid, genome mining, plant cell wall, fungi

Abbreviations

FA, ferulic acid; FAE, feruloyl esterase; SF, subfamily

Introduction

Esterified or etherified to polymers within the lignocellulosic matrix, ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) and to a lesser extent *p*-coumaric acid (4-hydroxycinnamic acid) are the most abundant hydroxycinnamic acids in plant cell walls [1, 2]. These hydroxycinnamic acids can be linked to arabinoxylans (*O*-5 position of α -L-arabinofuranosyl residues), which are the unique structural components in commelinid monocots (Family Poales, e.g. wheat, rice and barley). They can also be linked to neutral pectic side-chains of rhamnogalacturonan I (*O*-6 position of β -D-galactopyranosyl residues in (arabino)galactan, and *O*-2 or *O*-5 position of α -L-arabinofuranosyl residues in arabinan), which are mainly found in eudicotyledons (Order ‘core’ Caryophyllales, e.g. sugar beet) [3-8]. FA can form diferulic acids (mainly 5,5’-, 8-*O*-4’-, 8,5’-, 8,8’-diferulic acids) which cross-link two polysaccharide chains or a polysaccharide chain to lignin [7, 9-11]. Phenolic cross-links increase the physical strength and integrity of plant cell walls and reduce their biodegradability by microbial invaders and hydrolytic enzymes [2, 12].

Feruloyl esterases (or ferulic acid esterases, FAEs) [E.C. 3.1.1.73] represent a subclass of the carboxylic acid esterases (E.C. 3.1.1) and catalyze the hydrolysis of ester linkage between a phenolic acid and a poly- or oligosaccharide releasing hydroxycinnamic acids from plant cell wall polysaccharides [13, 14]. FAEs are able to release FAs and other phenolic acids from natural plant sources and agro-industrial byproducts. They facilitate the degradation of complex plant cell wall polysaccharides by removing the ester bonds between plant polymers providing accessibility for glycoside hydrolases and polysaccharide lyases [15-17]. Apart from being used as accessory

enzymes in the saccharification process, FAEs are also potential biocatalysts for synthesis of a broad range of novel bioactive components for use in the food, cosmetics and pharmaceutical industries [18, 19]. In 2014, an EU collaborative project ‘OPTIBIOCAT’ was granted by the 7th Framework Programme (FP7), which aims to use microbial esterases such as FAEs as biocatalysts for synthesis of potential antioxidants for cosmetic products [19]. As these enzymes are relevant for various industries, different types of FAE are required to fit specific conditions such as pH and temperature.

Recently, we reported a genome mining strategy for FAE discovery, in which more than 1,000 putative fungal FAE sequences were identified and, by using a phylogenetical analysis, classified into 13 subfamilies (SFs) [19]. In contrast to the high number of the putative FAE encoding genes, a limited number of fungal FAEs have been characterized in detail and they only cover SF1, 2, 5, 6, 7 and 13. To validate our genome mining strategy and expand the information on activity and properties of fungal FAEs, in this study we report the heterologous expression and biochemical characterization of selected recombinant FAEs discovered through genome mining, and covering the previously uncharacterized SFs.

Materials and methods

Bioinformatics

Genome mining and phylogenetic analysis were performed based on [19]. Signal peptides were predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>; [20]). The gene model correction of selected sequences was performed manually based on BlastX to identify and remove putative introns [21]. Sequence alignment was performed using Multiple Alignment using Fast

Fourier Transform (MAFFT) [22]. Theoretical molecular masses and pI were calculated by the ExPASy–ProtParam tool (<http://www.expasy.ch/tools/protparam.html> [23]).

Cloning of fae genes

The genes of the selected FAEs without signal peptide and introns were codon optimized and synthesized for expression in *P. pastoris* by NZYTech (Lisbon, Portugal). The gene products were digested by *PsiI* and *NotI* (Thermo Fisher Scientific), and cloned in frame with α -factor secretion signal in pPNic706 (ProteoNic, Leiden, the Netherlands). The obtained plasmids were purified from *Escherichia coli* DH5 α (Invitrogen), transformants selected on Luria Bertani medium supplemented with 50 μ g/mL kanamycin, fully sequenced (Macrogen, Amsterdam, the Netherlands), linearised by *SalI* (Thermo Fisher Scientific), and transformed into *P. pastoris* strain GS115 *his4* according to the manufacturer's recommendation.

Ten transformants were selected for the enzyme production screening, which was performed in 96 deep-well plates containing 0.8 mL medium. The selected clones were grown first in buffered minimal glycerol medium (1% yeast nitrogen base, 0.1 M potassium phosphate buffer pH 6.5, and 1% w/v glycerol). The plates were sealed with AeraSeal™ (Sigma Aldrich) and incubated overnight at 30°C, 900 rpm (INFORS HT Microtron, Bottmingen, Switzerland). A volume of cells equal to an OD₆₀₀ of 1.0 was harvested and resuspended in 0.8 mL buffered minimal methanol medium (1% yeast nitrogen base, 0.1 M potassium phosphate buffer pH 6.5, and 0.5 % methanol) for induction. The induction was performed at 30°C, 900 rpm for 72 h before being harvested. The cultures were supplemented with 80 μ L of 0.5% (v/v) methanol every 24 h.

Production and biochemical properties of recombinant FAEs

P. pastoris transformants were grown according to [24]. Induction was continued for 96 h at 28°C with methanol being supplemented to 0.5% (v/v) every 24 h. Culture supernatants were harvested (4000 x g, 4°C, 20 min), filtered (0.22 µm; Merck Millipore, Darmstadt, Germany) or concentrated (10 kDa cut off; Merck Millipore) and stored at -20°C prior further analysis. Molecular mass determination and deglycosylation were performed as previously described [24]. Protein concentrations were assessed from SDS-PAGE gels by densitometric method using ImageJ program [25] with bovine serum albumin (Pierce, Thermo Scientific) as a standard.

Enzyme activity assay of FAEs

Activity of the recombinant FAEs towards *p*NP-ferulate (Taros Chemicals, Dortmund, Germany) was performed in 275 µL reaction mixtures adapted from [26]. The *p*NP-ferulate substrate solution was prepared by mixing 10.5 mM *p*NP-ferulate (in dimethyl sulfoxide) and 100 mM potassium phosphate buffer, pH 6.5 containing 2.5% Triton-X (1:9, v/v). The reactions were performed in the presence of 250 µL *p*NP-ferulate substrate solution incubated with 25 µL of culture supernatant at 37°C. The release of *p*-nitrophenol was spectrophotometrically quantified by following the absorbance at 410 nm for 30 min with a 2 min interval and calculation according to [26]. All assays were performed in triplicate. One unit of FAE activity is defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol from *p*NP-ferulate per min under the assay conditions.

Activity towards methyl substrates [methyl caffeate, methyl ferulate, methyl *p*-coumarate, and methyl sinapate (Apin Chemicals Limited, Oxon, United Kingdom)] was assayed in 250 µL reaction mixtures according to [24] at 37°C for 5-30 min. Detection of substrates reduction was performed at 340 nm with a 2 min interval. The activity was determined from the standard curves

of the substrates (0.001-0.5 mM). Alternatively, the activities were assayed by HPLC (Agilent 1260 Infinity) using Kinetex 2.6u C18 100A column (Phenomenex). The quantification was performed by using calibration curves of the methyl substrates and their corresponding acids, and the detection was performed at 320 nm for methyl caffeate, methyl ferulate and methyl sinapate, and at 308 nm for methyl *p*-coumarate. The 1 mL reaction mixtures contained 850 μ L MOPS buffer (pH 6.0), 100 μ L enzyme and 50 μ L substrate (1 mM). The reactions were stopped by adding 1 vol 0.1% trifluoroacetic acid:acetonitrile solution (80:20). Chromatographic separation was performed by isocratic method with 80% 0.1% trifluoroacetic acid and 20% acetonitrile as solvents. The culture supernatant of *P. pastoris* harboring pPicZ α A plasmid without insert was used as negative control. All assays were performed in triplicate.

Enzyme activity assay of tannases

Methyl gallate (Sigma Aldrich, St. Louis, MO) was used for the assessment of tannase activity [27]. The reactions were performed in 125 μ L reaction mixtures containing 6.25 μ L of 100 mM methyl gallate stock solution (in dimethylformamide), 31.25 μ L of 100 mM phosphate buffer, pH 6.0, and 25 μ L water incubated with 62.5 μ L of culture supernatant at 30°C for 15 min. To detect the release of gallic acid, 75 μ L of 0.667% rhodanine (in methanol) (Sigma Aldrich) was added to the reaction mixture followed by 5 min incubation at 30°C, addition of 100 μ L of 0.5 M KOH, further incubation at 30°C for 5 min, and addition of 1 mL water prior to quantification by measuring the absorbance at 520 nm. The activity was determined from the standard curves of the substrates (0.006-0.6 mM). Alternatively, tannase activity was assayed by the HPLC method described above by detecting methyl gallate at 280 nm and using InfinityLab Poroshell 120 SB-AQ column (Agilent).

Enzyme activity assay of lipases

For assessment of the lipase activity, *p*NP-palmitate (Sigma Aldrich) was used as a substrate. Ten mM *p*NP-palmitate stock solution was prepared in isopropanol and diluted in 100 mM potassium phosphate buffer, pH 6.5. The reaction was performed in 1 mL reaction mixtures containing 900 µL of 0.2 mM *p*NP-palmitate in 100 mM potassium phosphate buffer, pH 6.5, and 100 µL of culture supernatant at 40°C. The release of *p*-nitrophenol was spectrophotometrically quantified by following the absorbance at 410 nm for 30 min with a 2 min interval and calculation according to [26].

Results and discussion

Discovery of novel fungal FAEs

A genome mining strategy was previously conducted by BLAST analysis with characterized FAEs against published fungal genomes to identify the putative fungal FAEs [19]. In the current study, four additional genomes from *Aspergillus* spp., i.e. *Aspergillus sydowii*, *Aspergillus wentii* DTO 134E9, *Aspergillus carbonarius* ITEM 5010 and *Aspergillus tubingensis* [28], were added to the analysis (A.1 in Supplementary materials). Over 1,000 putative fungal FAE sequences were identified, which were classified into 13 SFs. Of these, 33 FAE sequences (six previously characterized and 27 putative FAEs) from both ascomycetes and basidiomycetes, which covered five previously uncharacterized FAE subfamilies (SF3, 8, 9, 10, 12), were selected for biochemical characterization (Table 1). The enzymes from SF11 were not included in this study because they most likely possessed tannase activity.

Recombinant enzyme production of selected fungal FAEs

Heterologous expression of putative FAEs was performed using *P. pastoris* as a host organism. FAE sequences from both ascomycetes and basidiomycetes were produced as active enzymes in *P. pastoris*. Three of the selected enzymes, i.e. An09g05120 (*A. niger*), CsFae3 (*Ceriporiopsis subvermispota*) and ShFae1 (*Stereum hirsutum*), were produced at a concentration greater than 1,000 mg/L and five, namely GmFae1 (*Galerina marginata*), OrpFAE (*Orpinomyces* sp.), AnFaeL, An11g01220 (*A. niger*) and CsFae1 (*C. subvermispota*), were produced at a concentration greater than 100 mg/L. However, the production level of eight and 12 of the enzymes was less than 100 mg/L and 10 mg/L respectively, and five of them were not produced. Almost half of the recombinant enzymes showed higher molecular masses compared to the calculated ones, but the molecular mass reduced to the expected size after treatment with Endoglycosidase H indicating glycosylation by *P. pastoris*.

Activity and substrate preference among the selected fungal FAEs

To screen for FAE activity, *p*NP-ferulate was used as a substrate. Among 27 enzymes, 17 were active, with GmFae1 (*G. marginata*) showing the highest activity (Table 1). With respect to the substrate specificity, four methyl substrates (methyl *p*-coumarate, methyl caffeate, methyl ferulate, and methyl sinapate) were used. In agreement with the previous reports [29-31], AnFaeB (*A. niger*) and AsFaeH (*A. sydowii*) from SF1 hydrolyzed three methyl substrates: methyl *p*-coumarate, methyl caffeate and methyl ferulate (Table 2). Docking simulation of methyl sinapate on a structure of a member of SF1, AoFaeB (from *A. oryzae*, PDB: 3WMT), indicated that its narrow active site hindered the binding of the bulky structure of methyl sinapate [32]. AsFaeG (*A. sydowii*) from SF13 was also able to hydrolyze three methyl substrates similarly to the enzymes from SF1,

207 whereas the other FAEs from SF13 showed either low or no activity towards methyl ferulate. The
208 majority of the active FAEs from SF5 and SF6 hydrolyzed all four substrates, except FoFae2
209 (*Fusarium oxysporum* from SF6) and CcFae2 (*Coprinopsis cinerea* from SF6), which did not
210 hydrolyze methyl sinapate, and Settu1| 102085 (*Setosphaeria turcica* from SF5), which was only
211 active towards methyl caffeate and methyl sinapate. Because Settu1| 102085 as well as some other
212 esterases (see below) were not detected to hydrolyze methyl ferulate, they were referred to as
213 hydroxycinnamoyl esterases (HCE) instead of FAEs.

214
215 AnFaeA (*A. niger*) from SF7 hydrolyzed only methyl ferulate and methyl sinapate, which is
216 consistent with the previous reports [29, 31, 33]. The crystal structure of AnFaeA showed a long
217 and narrow cavity displaying hydrophobic residues that stabilize the aromatic moiety of the
218 substrate [34], and replacing the bulky aromatic residues (Tyr80 or Trp260) to smaller residues
219 broadened the substrate specificity of the enzyme [35].

220
221 Three FAEs from SF2, namely FoFaeC (*F. oxysporum*), AwFaeA (*A. wentii*) and GlFae1
222 (*Gymnopus luxurians*), showed no obvious substrate specificity pattern. FoFaeC hydrolyzed all
223 tested substrates with low activity on methyl sinapate, which is consistent with the previous report
224 [36]. AwFaeA also showed low activity towards methyl sinapate, but did not hydrolyze methyl *p*-
225 coumarate. GlFae1 showed the highest activity towards methyl sinapate and lower activity towards
226 methyl ferulate, but was not active towards methyl *p*-coumarate and methyl caffeate. The enzymes
227 from SF3 (Aspca3| 176503 from *A. carbonarius*), SF9 (Asptu1| 30001 from *A. tubingensis*) and
228 SF12 (Galma1| 254175 from *G. marginata*) were not active towards methyl ferulate and *p*NP-
229 ferulate, but showed activity towards only one methyl substrate with no specific pattern. Therefore,

these esterases were also referred to as HCEs. The enzyme from SF10 (OrpFAE from *Orpinomyces* sp.) was not active towards methyl substrates, but showed activity towards *p*NP-ferulate.

Previously, members of SF1, 2, 5, 6, 7 and 13 were shown to possess FAE activity [19]. In the present study, we reported that enzymes from SF8, 9 and 10 showed limited activity towards synthetic FAE substrates, as well as towards methyl gallate for SF9. Recently, an esterase from *Auricularia auricularia-judae* (EstBC), belonging to SF8 was described that acted efficiently on both artificial cinnamic and benzoic acid esters, but was not active on complex natural FAE substrates [37]. Hence, the enzymes from these subfamilies should be further tested towards feruloylated saccharides or natural substrates, e.g. wheat bran or sugar beet pectin, to confirm their true FAE activity.

Enzymes with other activity

Five enzymes (CsTan1 – *C. subvermispora*, AnFaeL – *A. niger*, AsFaeL – *A. sydowii*, An11g01220 – *A. niger*, An09g05120 – *A. niger*) were active towards methyl gallate. These enzymes belong to SF9, SF10 and SF13, indicating that the representatives of these SFs may be tannases. Recently, two FAEs from *Schizophyllum commune* were reported to hydrolyse methyl gallate [38]. It is possible that these enzymes are the bridge in the evolution from tannases to FAEs or *vice versa*. Surprisingly, one enzyme (Aspca3| 176503 from *A. carbonarius*) was active towards *p*NP-palmitate, although this enzyme does not share amino acid sequence similarity to known lipases.

Conclusions

In the present study, we have confirmed the ability of the genome mining strategy to identify fungal FAE encoding genes, by demonstrating that 20 out of 27 putative fungal FAEs possessed FAE activity towards *p*NP-ferulate and/or methyl hydroxycinnamate substrates. Previously, members of SF1, 2, 5, 6, 7 and 13 were shown to possess FAE activity [19]. In the present study, we also showed that the enzymes from SF8, 9 and 10 are active towards the synthetic FAE substrates. However, it should be noted that most of the enzymes from SF9 possessed tannase activity. Additional experiments are needed to confirm whether the enzymes of these subfamilies are true FAEs. The selected esterases from SF3 and SF12 were active towards methyl caffeate and methyl *p*-coumarate, respectively, but not towards methyl ferulate. In comparison with the previously characterized fungal FAEs, most of the new FAEs showed similar levels of specific activity. Thus, they may potentially be eligible candidates for related biotechnological applications.

Funding This work was supported by the European Union, Grant agreement no: 613868 (OPTIBIOCAT).

Acknowledgments

The authors would like to thank Dr. Laura Iancu (DuPont Industrial Biosciences, Wageningen, the Netherlands) for providing the lipase assay method.

Appendix A. Supplementary data

Table A.1 Sequences of putative FAEs from four aspergilli.

References

- [1] Kroon PA, Garcia-Conesa MT, Fillingham IJ, Hazlewood GP, Williamson G. Release of ferulic acid dehydrodimers from plant cell walls by feruloyl esterases. *J Sci Food Agr* 1999;79:428-434.
- [2] de O. Buanaфина MM. Feruloylation in grasses: current and future perspectives. *Mol Plant* 2009;2:861-872.
- [3] Carpita NC. Structure and biogenesis of the cell walls of grasses. *Annu Rev Plant Physiol Plant Molec Biol* 1996;47:445-476.
- [4] Harris PJ. Diversity in plant cell walls. In: Henry RJ (editor). *Plant diversity and evolution: genotypic and phenotypic variation in higher plants*, Oxon: CABI Publishing; 2005, p. 201-228.
- [5] Scheller HV, Ulvskov P. Hemicelluloses. *Annu Rev Plant Biol* 2010;61:263-289.
- [6] Harris PJ, Trethewey JAK. The distribution of ester-linked ferulic acid in the cell walls of angiosperms. *Phytochem Rev* 2010;9:19-33.
- [7] Levigne SV, Ralet MCJ, Quemener BC, Pollet BNL, Lapierre C, Thibault JFJ. Isolation from sugar beet cell walls of arabinan oligosaccharides esterified by two ferulic acid monomers. *Plant Physiol* 2004;134:1173-1180.
- [8] Ralet MC, Thibault JF, Faulds CB, Williamson G. Isolation and purification of feruloylated oligosaccharides from cell walls of sugar-beet pulp. *Carbohydr Res* 1994;263:227-241.
- [9] Waldron KW, Parr AJ, Ng A, Ralph J. Cell wall esterified phenolic dimers: Identification and quantification by reverse phase high performance liquid chromatography and diode array detection. *Phytochem Anal* 1996;7:305-312.
- [10] Levigne S, Ralet MC, Quemener B, Thibault JF. Isolation of diferulic bridges ester-linked to arabinan in sugar beet cell walls. *Carbohydr Res* 2004;339:2315-2319.

298 [11] Iiyama K, Lam TBT, Stone BA. Covalent cross-links in the cell-wall. *Plant Physiol*
299 1994;104:315-320.

300 [12] de Oliveira DM, Finger-Teixeira A, Rodrigues Mota T, Salvador VH, Moreira-Vilar FC,
301 Correa Molinari HB, *et al.* Ferulic acid: a key component in grass lignocellulose recalcitrance to
302 hydrolysis. *Plant Biotechnol J* 2015;13:1224-1232.

303 [13] de Vries RP, Visser J. *Aspergillus* enzymes involved in degradation of plant cell wall
304 polysaccharides. *Microbiol Mol Biol Rev* 2001;65:497-522.

305 [14] Wong DWS. Feruloyl esterase - A key enzyme in biomass degradation. *Appl Biochem*
306 *Biotechnol* 2006;133:87-112.

307 [15] Tabka MG, Herpoël-Gimbert I, Monod F, Asther M, Sigoillot JC. Enzymatic
308 saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and
309 feruloyl esterase treatment. *Enzyme Microb Technol* 2006;39:897-902.

310 [16] Gottschalk LMF, Oliveira RA, Bon EPdS. Cellulases, xylanases, β -glucosidase and ferulic
311 acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of
312 sugarcane bagasse. *Biochem Eng J* 2010;51:72-78.

313 [17] de Vries RP, Kester HCM, Poulsen CH, Benen JAE, Visser J. Synergy between enzymes
314 from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. *Carbohydr Res*
315 2000;327:401-410.

316 [18] Topakas E, Vafiadi C, Christakopoulos P. Microbial production, characterization and
317 applications of feruloyl esterases. *Process Biochem* 2007;42:497-509.

318 [19] Dilokpimol A, Mäkelä MR, Aguilar-Pontes MV, Benoit-Gelber I, Hildén KS, de Vries RP.
319 Diversity of fungal feruloyl esterases: updated phylogenetic classification, properties, and
320 industrial applications. *Biotechnol Biofuels* 2016;9:231.

321 [20] Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal
 322 peptides from transmembrane regions. *Nat Meth* 2011;8:785-786.

323 [21] Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schäffer AA, *et al.* Protein
 324 database searches using compositionally adjusted substitution matrices. *FEBS J* 2005;272:5101-
 325 5109.

326 [22] Katoh K, Standley DM. MAFFT Multiple Sequence Alignment Software Version 7:
 327 Improvements in performance and usability. *Mol Biol Evol* 2013;30:772-780.

328 [23] Gasteiger E, Hoogland C, Gattiker A, Duvaud Se, Wilkins MR, Appel RD, *et al.* Protein
 329 identification and analysis tools on the ExPASy Server. In: Walker JM (editor). *The Proteomics*
 330 *Protocols Handbook*, Totowa, NJ: Humana Press; 2005, p. 571-607.

331 [24] Dilokpimol A, Mäkelä MR, Mansouri S, Belova O, Waterstraat M, Bunzel M, *et al.*
 332 Expanding the feruloyl esterase gene family of *Aspergillus niger* by characterization of a feruloyl
 333 esterase, FaeC. *N Biotechnol* 2017;37, Part B:200-209.

334 [25] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image
 335 analysis. *Nat Meth* 2012;9:671-675.

336 [26] Mastihuba Vr, Kremnický Lr, Mastihubová M, Willett JL, Côté GL. A spectrophotometric
 337 assay for feruloyl esterases. *Anal Biochem* 2002;309:96-101.

338 [27] Sharma S, Bhat TK, Dawra RK. A spectrophotometric method for assay of tannase using
 339 rhodanine. *Anal Biochem* 2000;279:85-89.

340 [28] de Vries RP, Riley R, Wiebenga A, Aguilar-Osorio G, Amillis S, Uchima CA, *et al.*
 341 Comparative genomics reveals high biological diversity and specific adaptations in the
 342 industrially and medically important fungal genus *Aspergillus*. *Genome Biol* 2017;18:28.

343 [29] Kroon PA, Faulds CB, Brézillon C, Williamson G. Methyl phenylalkanoates as substrates to
344 probe the active sites of esterases. *Eur J Biochem* 1997;248:245-251.

345 [30] Levasseur A, Benoit I, Asther M, Asther M, Record E. Homologous expression of the
346 feruloyl esterase B gene from *Aspergillus niger* and characterization of the recombinant enzyme.
347 *Protein Expr Purif* 2004;37:126-133.

348 [31] Benoit I, Navarro D, Marnet N, Rakotomanomana N, Lesage-Meessen L, Sigoillot J-C, *et*
349 *al.* Feruloyl esterases as a tool for the release of phenolic compounds from agro-industrial by-
350 products. *Carbohydr Res* 2006;341:1820-1827.

351 [32] Suzuki K, Hori A, Kawamoto K, Thangudu RR, Ishida T, Igarashi K, *et al.* Crystal structure
352 of a feruloyl esterase belonging to the tannase family: A disulfide bond near a catalytic triad.
353 *Proteins: Struct, Funct, Bioinf* 2014;82:2857-2867.

354 [33] Faulds CB, Williamson G. Purification and characterization of a ferulic acid esterase (FAE-
355 III) from *Aspergillus niger*: specificity for the phenolic moiety and binding to microcrystalline
356 cellulose *Microbiol-Sgm* 1994;140:779-787.

357 [34] Hermoso JA, Sanz-Aparicio J, Molina R, Juge N, González R, Faulds CB. The crystal
358 structure of feruloyl esterase A from *Aspergillus niger* suggests evolutive functional convergence
359 in feruloyl esterase family. *J Mol Biol* 2004;338:495-506.

360 [35] Faulds CB, Molina R, Gonzalez R, Husband F, Juge N, Sanz-Aparicio J, *et al.* Probing the
361 determinants of substrate specificity of a feruloyl esterase, AnFaeA, from *Aspergillus niger*.
362 *FEBS J* 2005;272:4362-4371.

363 [36] Moukouli M, Topakas E, Christakopoulos P. Cloning, characterization and functional
364 expression of an alkalitolerant type C feruloyl esterase from *Fusarium oxysporum*. *Appl*
365 *Microbiol Biotechnol* 2008;79:245-254.

366 [37] Haase-Aschoff P, Linke D, Nimtz M, Popper L, Berger RG. An enzyme from *Auricularia*
367 *auricula-judae* combining both benzoyl and cinnamoyl esterase activity. *Process Biochem*
368 2013;48:1872-1878.

369 [38] Nieter A, Kelle S, Linke D, Berger RG. Feruloyl esterases from *Schizophyllum commune* to
370 treat food industry side-streams. *Bioresour Technol* 2016;220:38-46.

371

Table 1 Molecular mass, production level and specific activity (towards *p*NP-ferulate) of characterized FAEs in this study^a.

Fungal species	Phylum ^b	Accession number	SF ^c	Name ^d	Calculated molecular mass (kDa)	Apparent molecular mass (kDa) ^e	Calculated pI	Production (mg/L)	Specific activity ^f (mU/mg)	Remark ^g
<i>Aspergillus niger</i>	Asco	Q8WZI8.1	1	AnFaeB	55.8	100 (55)**	4.95	55	2	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 293049	1	AsFaeB	55.4	55*	4.89	<1	4	
<i>Fusarium oxysporum</i>	Asco	jgi Fusox1 5438	2	FoFaeC	59.4	70 (61)**	6.89	6	Active	
<i>Aspergillus wentii</i>	Asco	jgi Aspwe1 156253	2	AwFaeJ	56.0	57	4.99	<1	Active	
<i>Gymnopus luxurians</i>	Asco	jgi Gymlu1 46632	2	GlFae1	56.8	58	4.53	3	na	
<i>Aspergillus carbonarius</i>	Asco	jgi Aspca3 176503	3	-	56.1	58	5.02	4	na	Lipase activity
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 901052	3	-	55.8	nd	4.88	np	na	
<i>Aspergillus nidulans</i>	Asco	EAA62427.1	5	AnidFAEC	25.8	30 (30)**	4.47	30	2	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 154482	5	AsFaeC	25.9	30 (30)**	4.44	15	2	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 48859	5	AsFaeE	26.6	nd	3.93	np	na	
<i>Setosphaeria turcica</i>	Asco	jgi Settu1 102085	5	-	26.9	26	8.90	<1	na	
<i>Myceliophthora thermophila</i> (<i>Sporotrichum thermophile</i>)	Asco	AEO62008.1	6	MtFae1a	29.5	35 (30)**	4.44	45	3	
<i>Fusarium oxysporum</i>	Asco	jgi Fusox1 8990	6	FoFae2	29.6	30	8.44	4	Active	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 1158585	6	AsFaeG	29.5	32*	4.24	7	4	

<i>Stagonospora nodorum</i>	Asco	jgi Stano2 8578	6	-	31.3	30	5.16	12	na	
<i>Coprinopsis cinerea</i>	Basidio	jgi Copci1 3628	6	CcFae2	37.7	38	6.63	<1	Active	
<i>Ceriporiopsis subvermispora</i>	Basidio	jgi Cersu1 68569	6	CsFae1	36.9	70	4.58	280	7	
<i>Galerina marginata</i>	Basidio	jgi Galma1 144217	6	GmFae1	35.2	42	5.83	600	120	
<i>Aspergillus niger</i>	Asco	CAA70510	7	AnFaeA	28.6	40 (35)**	4.19	55	4	
<i>Aspergillus clavatus</i>	Asco	jgi Aspcl1 3045	8	-	39.1	40*	6.34	51	na	
<i>Ceriporiopsis subvermispora</i>	Basidio	jgi Cersu1 89153	9	CsTan1	55.0	90 (60)***	4.59	np	na	Tannase activity
<i>Aspergillus tubingensis</i>	Asco	jgi Asptu1 30001	9	-	57.6	57	4.50	<1	na	
<i>Aspergillus niger</i>	Asco	An15g05280	9	AnFaeL	58.6	68 (60)***	4.93	500	na	Tannase activity
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 41271	9	AsTanA	57.6	60*	4.99	32	na	Tannase activity
<i>Orpinomyces</i> sp.	Neo	AAF70241.1	10	OrpFAE	59.0	68	5.06	600	10	
<i>Aspergillus niger</i>	Asco	An11g01220	10	-	55.0	88	4.11	500	na	Tannase activity
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 194109	10	-	52.7	nd	4.67	np	na	
<i>Dichomitus squalens</i>	Basidio	jgi Dicsq1 136925	12	-	56.3	nd	4.78	np	na	
<i>Galerina marginata</i>	Basidio	jgi Galma1 254175	12	-	56.0	59	6.90	3	na	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 160668	13	AsFaeK	59.4	55*	4.90	<1	4	
<i>Stereum hirsutum</i>	Basidio	jgi Stehi1 73641	13	ShFae1	58.1	67	4.40	2,160	13	
<i>Aspergillus niger</i>	Asco	An09g05120	13	-	53.1	66	4.79	3,400	na	Tannase activity

<i>Ceriporiopsis subvermispora</i>	Basidio	jgi Cersu1 150639	13	CsFae2	55.1	nd	4.28	2,400	1
--	---------	-----------------------	----	--------	------	----	------	-------	---

^a na, no activity detected; nd, no protein band detected; np, no protein produced or the protein level was lower than detection limit.

^b Asco, ascomycete; Basidio, basidiomycete; Neo, Neocallimastigomycete

^c According to [19]

^d Name in bold indicates the previously reported FAEs [19]. The abbreviation of the enzyme code is based on the convention protein names for different species and not on the types of FAEs.

^e * indicates the protein band was visible only after deglycosylation by Endoglycosidase H. ** indicates molecular mass after deglycosylation by Endoglycosidase H, *** indicates molecular mass after deglycosylation by PNGase F.

^f One unit of enzyme activity is defined as the amount of enzyme releasing 1 μ mol of *p*-nitrophenol from *p*NP-ferulate per min under assay conditions. Active indicates the enzyme was active but the specific activity could not be calculated.

^g Tannase and lipase activities were evaluated using methyl gallate and *p*NP-palmitate as substrate, respectively

Table 2 Relative FAE activity towards four methyl substrates^a.

Fungi	SF ^b	Name ^c	Relative activity (%) ^d			
			Methyl <i>p</i> -coumarate	Methyl caffeate	Methyl ferulate	Methyl sinapate
<i>Aspergillus niger</i>	1	AnFaeB	100	77	59	na
<i>Aspergillus sydowii</i>	1	AsFaeB	100	66	60	na
<i>Fusarium oxysporum</i>	2	FoFaeC	100	67	29	9
<i>Aspergillus wentii</i>	2	AwFaeJ	na	100	88	29
<i>Gymnopus luxurians</i>	2	GlFae1	na	na	18	100
<i>Aspergillus carbonarius</i>	3	-	na	100	na	na
<i>Aspergillus nidulans</i>	5	AnidFAEC	98	42	100	46
<i>Aspergillus sydowii</i>	5	AsFaeC	91	45	100	40
<i>Aspergillus sydowii</i>	5	AsFaeE	na	na	Low*	na
<i>Setosphaeria turcica</i>	5	-	na	100	na	91
<i>Myceliophthora thermophila</i>	6	MtFae1a	100	77	90	36
<i>Fusarium oxysporum</i>	6	FoFae2	100	60	16	na
<i>Aspergillus sydowii</i>	6	AsFaeG	95	53	100	60
<i>Stagonospora nodorum</i>	6	-	na	na	na	na
<i>Coprinopsis cinerea</i>	6	CcFae2	100	41	23	na
<i>Ceriporiopsis subvermispota</i>	6	CsFae1	na	na	na	na
<i>Galerina marginata</i>	6	GmFae1	Low*	Low*	Low*	Low*
<i>Aspergillus niger</i>	7	AnFaeA	na	na	100	81
<i>Aspergillus clavatus</i>	8	-	na	na	Low*	na
<i>Aspergillus tubingensis</i>	9	-	na	100	na	na
<i>Aspergillus niger</i>	9	AnFaeL	na	Low*	Low*	Low*
<i>Aspergillus sydowii</i>	9	AsFaeL	na	na	na	na
<i>Orpinomyces</i> sp.	10	OrpFAE	na	na	na	na
<i>Aspergillus niger</i>	10	-	Low*	Low*	Low*	Low*
<i>Galerina marginata</i>	12	-	100	na	na	na
<i>Aspergillus sydowii</i>	13	AsFaeK	100	65	60	na
<i>Stereum hirsutum</i>	13	ShFae1	na	na	Low*	na
<i>Aspergillus niger</i>	13	-	na	Low*	Low*	Low*
<i>Ceriporiopsis subvermispota</i>	13	CsFae2	na	na	na	na

^a na, no activity detected^b According to [19].^c The abbreviation of the enzyme code is based on the conventional protein names for different species and not on the types of FAEs. Name in bold indicates the previously reported FAEs^d The relative activity was calculated as a percentage of the highest activity for each enzyme that was set to 100%, Low* indicates activity which was lower than the reliable detectable range